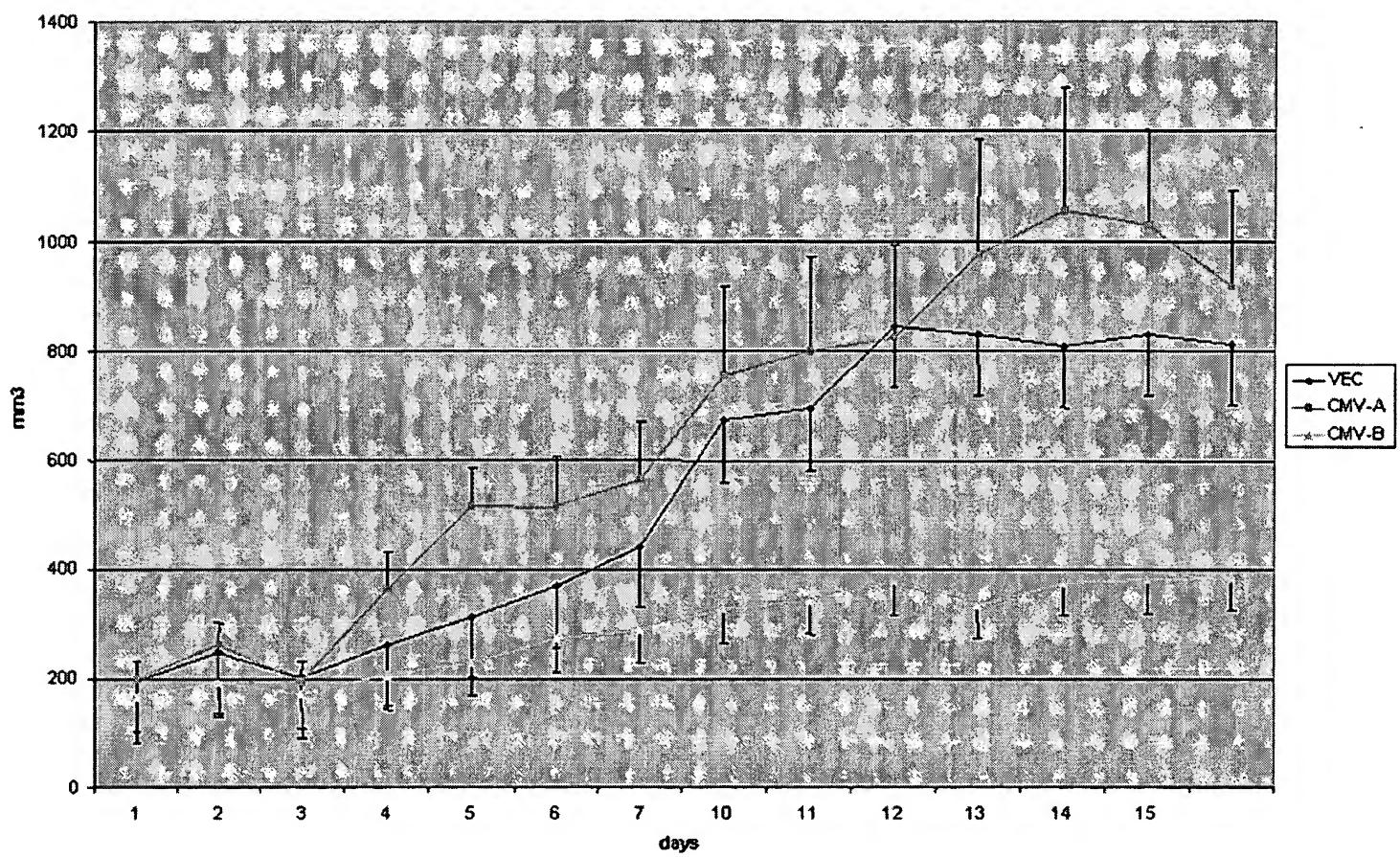


## **Exhibit B**

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# Transcriptional Targeting in Cancer Gene Therapy

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Cancer gene therapy has been one of the most exciting areas of therapeutic research in the past decade. In this review, we discuss strategies to restrict transcription of transgenes to tumour cells. A range of promoters which are tissue-specific, tumour-specific, or inducible by exogenous agents are presented. Transcriptional targeting should prevent normal tissue toxicities associated with other cancer treatments, such as radiation and chemotherapy. In addition, the specificity of these strategies should provide improved targeting of metastatic tumours following systemic gene delivery. Rapid progress in the ability to specifically control transgenes will allow systemic gene delivery for cancer therapy to become a real possibility in the near future.

## INTRODUCTION

Gene therapy is the transfer of exogenous genes, called transgenes, into the somatic cells of a patient to obtain a therapeutic effect. Initially, gene therapy was viewed as an approach for treating hereditary diseases, but its potential role in the treatment of acquired diseases such as cancer is now widely recognised. In theory, gene therapy offers the potential for enormous improvements in the targeting of cancer therapy, but it is clear that this has not yet been achieved, although early trials are promising.

One of the main goals for all cancer therapies is the selective targeting and killing of tumour cells, thereby increasing the therapeutic ratio. Both chemotherapy and radiotherapy induce dose limiting normal tissue toxicities, which reduce their clinical effectiveness. Cancer gene therapy has the advantage that normal tissue toxicity might be avoided if suitable strategies can be employed to target the therapeutic transgene directly to tumour cells; an outcome that conventional therapeutic approaches have failed to achieve.

To date, clinical trials have focused on the delivery of genes directly to the tumour site by intratumoural injection using both viral and nonviral delivery agents, thereby largely avoiding normal tissues. However, the goal of most cancer gene therapy is to be able to administer a suitably packaged transgene systemically and achieve a high level of tumour targeting. This will be important for targeting the majority of tumours, that are not accessible for direct injection, and to ensure adequate distribution of the transgene throughout the tumour mass. The blood supply still offers the best opportunity to do this. Systemic delivery of transgenes would also allow targeting of both the primary tumour and metastatic deposits, which must be controlled if therapy is to be successful. A number of

strategies are now being developed to target both viral and nonviral delivery agents to tumour cells. These include exploitation of natural viral tropisms, such as those exhibited by adenoviruses to target lung epithelium; retargeting viruses using a bispecific molecule to simultaneously block native receptor binding, redirecting virus to a tissue-specific receptor; genetically modifying the virus to ablate native receptor interactions and incorporating a novel ligand into one of the virus' coat proteins; using tissue-specific ligands or monoclonal antibodies incorporated onto the surface of liposomes to direct them to target cells. A detailed description of these targeted delivery strategies, lies beyond the scope of this review (for reviews covering this area see references [1, 2, 3, 4]).

As well as controlling the delivery of the therapeutic gene to the tumour tissue, controlled regulation of transgene expression is now playing a major role in targeted cancer gene therapy strategies. Indeed, by combining targeted delivery with tumour-specific expression, the level of transgene product in nontarget normal tissues, compared with that in tumours, can be greatly reduced. The purpose of this review is to focus on transcriptional targeting of transgenes, which will undoubtedly be an essential component of systemic cancer gene therapy.

## TRANSCRIPTIONAL CONTROL OF EXPRESSION

In the last decade, it has become increasingly clear that gene expression is regulated by a complex interplay of factors that function in a cell-type-specific manner to produce diverse effects. These subtleties arise from the balance of tissue-specific transcriptional control elements present in the various cell types, for example, hepatic, melanocyte, neuronal, and erythroid or tumour-specific promoters/enhancers that are activated

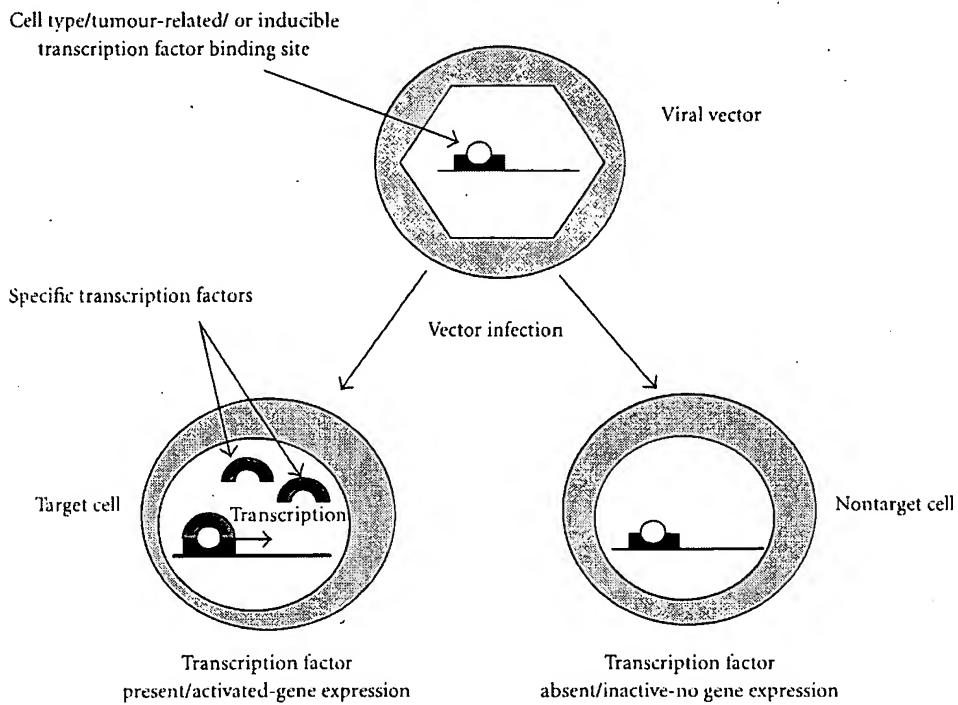


FIGURE 1. The use of specific tissue/tumour/inducible promoters to allow targeted transcription in tumour cells. Tissue-specific, tumour-specific, or inducible (eg, by radiation, drugs, etc) promoters can limit gene expression to target cells which express a specific transcription factor, or in which the transcription factors are activated exogenously.

in diseased states, or as a result of being exposed to an unfavourable tumour-associated microenvironment, for example, hypoxia. These *cis*-acting elements can be harnessed to drive the transcription of a therapeutic gene in a tissue- or tumour-specific manner (Figure 1). The use of tissue-specific cellular promoters to restrict transgene expression usually results in constitutive expression in the target tissue. However, for some therapeutic strategies, it will be preferable to regulate the duration and level of expression exogenously. This may be achieved by the use of cellular promoters that are preferentially activated under certain conditions to drive transgene expression in the target cell population. Research has focused on promoters induced by ionising radiation, heat, and small molecules. The use of tissue- and tumour-specific and exogenously controlled inducible promoters will now be discussed, as well as novel molecular adaptations of these promoters to strengthen gene expression. However, because the number of promoters that have been used for transcriptional targeting of transgenes for cancer gene therapy is very large, it is not feasible to discuss all of these in detail. The available data to date will therefore be presented in a series of tables.

#### TISSUE-SPECIFIC PROMOTERS

Transcriptional targeting utilising tissue-specific promoters exploits genes that are switched on only in certain tissues. A list of tissue-specific promoters currently being

used in cancer gene therapy is given in Table 1. One of the main limitations of this type of promoter is that transgene expression may lead to cytotoxic effects in normal as well as tumour tissue derived from that same cell type. Therefore, the use of such promoters must be restricted to tissues in which damage is not critical for the survival of the host, for example, prostate, melanocytes, or thyroid. If the tissue/organ is critical, then the transgenes must be delivered directly to the tumour site to prevent normal tissue toxicity or be delivered with retroviruses which are less efficient at infecting nondividing slowly proliferating normal tissues, such as the liver.

#### *Melanocyte-restricted expression*

The use of tissue-specific promoters was pioneered by Vile and Hart in 1993, with the use of the tyrosinase promoter to target melanocytes/melanomas. Melanin biosynthesis is restricted to melanocytes, partly, as a consequence of transcriptional regulation of gene products, such as tyrosinase or tyrosinase-related protein 1, involved in this biochemical pathway. Vile and Hart demonstrated that small elements of the 5' flanking regions of these two genes could drive reporter gene expression in murine and human melanoma cell lines *in vitro* and *in vivo*, but they could detect only low level expression in other cell types *in vitro* and no expression in Colo 26 tumours grown *in vivo* following direct injection of the transgenes [5]. They went on to demonstrate efficacy *in vivo* using the herpes simplex thymidine kinase (HSV-

TABLE 1. Tissue-specific promoters used in cancer gene therapy.

Promoter	Target tissue/tumour	Reference
Tyrosinase	Melanocytes/melanoma	[5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]
Prostate-specific antigen (PSA)	Prostate	[19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33]
Prostate-specific membrane antigen (PSMA)	Prostate/also targets vascular endothelium of other tumours	[31, 34, 35, 36]
Probasin	Prostate	[28, 37, 38, 39, 40, 41]
Human glandular kallikrein (hK2)	Prostate	[25, 37, 38, 42]
Glial fibrillary acidic protein (GFAP)	Glial/glioma	[43, 44, 45, 46, 47, 48, 49, 50, 51]
Myelin basic protein (MBP)	Glial and astrocytes/glioma	[43, 44, 52, 53, 54, 55]
Myelin proteolipid protein	Glial/glioma	[43, 44]
Neural specific enolase	Neuronal/SCLC	[48, 56]
Neuronal specific synapsin I	Neuronal	[57, 58]
Ncx/Hox11L.1	Neural crest derived cells/neuroblastoma	[59]
Albumin	Liver/hepatocellular carcinoma	[60, 61, 62, 63, 64, 65, 66]
Surfactant protein B	Type II alveolar and bronchial cells/lung cancer	[67, 68]
Thyroglobulin	Thyroid/thyroid carcinomas	[69, 70, 71, 72, 73]
Ovarian-specific promoter	Ovarian	[74, 75]

*tk*)/gancyclovir (GCV) combination, which gave a significant tumour growth delay following intratumoural injection of the tyrosinase-driven constructs and decreased metastatic potential in mice injected with melanoma cells expressing these constructs following treatment with GCV [6]. In a separate study, tissue-specific expression of IL-2, IL-4, and GM-CSF was also seen in vitro and in vivo [7]. As an extension to this work, retroviruses were developed containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of HSV-*tk* or IL-2 genes [8, 9]. Antitumour efficacy and tissue specificity were observed after intratumoural and IV delivery using localised and metastatic melanoma models [7]. Other studies have demonstrated antitumour efficacy using both retrovirus [10] and cationic liposomes [11] to achieve tyrosinase driven expression of cytosine deaminase (CD), which in turn catalyses the conversion of the nontoxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Adenoviral delivery of the murine tyrosinase promoter, introduced into the E1 region, has been used to drive melanoma-specific expression of the reporter gene  $\beta$ -galactosidase [12]. Both studies showed effective transcriptional targeting. More recently, tissue-specific elements of the tyrosinase promoter have been incorporated into more elaborate DNA constructs, that incorporate either a second tumour-specific promoter, cyclin A [13] for added specificity, or elements to help increase tyrosinase promoter expression [14]. As an extension to this, both targeted delivery and transcriptional targeting of melanoma cells via the tyrosinase promoter

were combined in one study. Anionic liposomes carrying a peptide ligand for targeting  $\alpha$ -v $\beta$ 3-integrin receptors, commonly upregulated on malignant melanoma cells, were used in this instance for the transductional targeting [15]. These more elaborate combined approaches will be discussed in detail later as future perspectives.

#### Prostate-specific expression

Another well-characterised tissue-specific promoter is that of prostate-specific antigen (PSA). Prostate-specific antigen is expressed predominantly in the prostate and is transcriptionally upregulated by androgens. Pang et al [19] demonstrated that elements from the 5' region of this gene can drive reporter gene expression in the PSA-producing cell line, LNCaP, but not in the nonproducing tumour cell lines, DU145 and PC-3, nor in renal or breast cancer cell lines. Tumour cell growth was inhibited when the PSA promoter was used to drive expression of antisense constructs targeting DNA polymerase  $\alpha$  and topoisomerase  $\alpha$  in prostate tumour cell lines, but not in cell lines of any other tumour [20]. In addition, a replication-competent adenovirus, CN706, has been developed with a selective toxicity for PSA-positive prostate cancer cells, using minimal enhancer/promoter constructs derived from the 5' flank of the PSA gene, to drive the E1A gene [21]. A single intratumoural injection of the virus destroyed large LNCaP tumours and abolished PSA production in mice. Gotoh et al [22] have developed an attenuated adenovirus which expresses HSV-*tk* from a 5837-bp fragment of the PSA promoter. This

promoter was active in both androgen-dependent and independent PSA-producing prostate cancer cells in vitro, and in prostate tumours in castrated hosts. Chimeric constructs which have also been engineered improved the activity and specificity of the PSA enhancer. Importantly, intravenous delivery of adenoviral constructs, with either duplication of the enhancer core or insertion of tandem copies of the proximal androgen response elements, not only showed enhanced activity and inducibility but also retained tissue discrimination for human LAPC-9 tumour xenografts [23]. Constructs utilising PSA promoter elements have also been developed to drive the expression of the sodium iodide transporter to concentrate radioiodine in the prostate [24]. They have also been used to increase the expression of nitroreductase or CD allowing sensitisation of prostate cells to the prodrugs, CB1954 [25] or 5-FC, respectively [76]. PSA promoter driven transgenes have also been delivered using an HIV-1-based lentiviral vector [26] and liposomes [27].

Promoters of prostate-specific genes other than PSA are now being utilised. Prostate-specific membrane antigen (PSMA) is a type-2 membrane protein expressed in virtually all prostate cancers and their metastases. Moreover, unlike PSA, PSMA expression is upregulated by androgen deprivation. These advantages make PSMA a useful target for advanced prostate cancer therapy, especially in combination with conventional hormonal treatment. O'Keefe et al [34] have determined the most active regions of the PSMA enhancer and revealed a 1.6-kbp region which was active in driving prostate-specific CD expression, sensitising cells in vitro to 5-FC more than 50-fold compared to control cells. Furthermore, C4-2 prostate tumours grown in nude mice were eliminated by 5-FC when CD expression was induced by the PSMA promoter/enhancer [35]. This type of promoter will be particularly useful for patients who have undergone androgen ablation therapy and are suffering from a relapse of the disease.

Human glandular kallikrein (hK2) and PSA are related members of the human kallikrein gene family, however, unlike PSA whose expression displays an inverse correlation with prostate cancer stage and grade, hK2 is upregulated in higher grade and stage of the disease. Yu et al [37] identified an androgen-dependent and prostate-specific enhancer located between -3.4 and -5.2 kbp upstream of the hK2 transcription start site. A replication-competent adenovirus was constructed, CV763, in which the E1A gene was driven by the hK2 enhancer/promoter. Specificity for prostate tumours *in vivo* was impressive. More recently, a recombinant adenovirus expressing EGFP under the control of a triplicate hK2 enhancer/promoter led to robust tumour-restricted EGFP expression [38].

Probasin is another prostate-specific promoter, which has been used by many groups for transcriptional targeting of therapeutic transgenes. Steiner et al [28] used probasin to drive the expression of the *LacZ* reporter gene in a canine model following intraprostatic injection. They

were able to demonstrate that, although adenovirus was detected in other tissue, *lacZ* mRNA could be detected by RT-PCR only in the prostate and not in other tissues. A small potent composite rat probasin promoter has been developed, ARR(2)PB, which is both androgen- and glucocorticoid-inducible [39]. When used to drive cell cycle-independent caspases, in an adenoviral system, it can lead to apoptosis in prostate cancer xenografts, but not in xenografts of other tumours, when the dimeric ligand AP20187 is delivered to trigger caspase activation [77]. More importantly, systemic delivery of a similar adenovirus, utilising the ARR(2)PB composite promoter to drive *BAX* expression, revealed no toxicity in the liver, lung, kidney, or spleen, but resulted in highly specific cytotoxicity in the prostatic cancer cell line LNCaP [40]. Lastly, a tumour-specific rather than prostate-specific promoter from the osteocalcin gene has shown a considerable promise in targeting prostate cancer bone metastases. This will be discussed in the next section.

### **Glial-restricted expression**

A number of tissue-specific promoters have now been tested for targeting of gliomas. In 1993, Miyao et al [43], described 2.5 kbp, 1.3 kbp, and 1.5 kbp 5' flanking regions of the mouse glial fibrillary acidic protein (GFAP) gene, myelin basic protein (MBP) gene, and myelin proteolipid protein (PLP) gene, respectively, which conferred specificity for glial cells. Following retroviral delivery, transcription was initiated more effectively in glioma cells via the MBP promoter where the HSV-*tk* transgene sensitised cells to gancyclovir in both murine and human glioma cells [44]. GFAP promoter driven expression of HSV-*tk* and therapeutic efficacy for gliomas was demonstrated by other groups [45, 46]. The RL1 variant herpes simplex virus 1 (HSV-1) has also been engineered to target intracerebral tumours of glial and astrocyte origin by using the GFAP promoter. These viruses, 1774 and 1775, showed high levels of  $\beta$ -galactosidase expression specifically in astrocytes following intracerebral inoculation [47]. Replication-defective adenoviral vectors have also been engineered to contain either the GFAP promoter or the neuronal specific enolase promoter [48, 49]. Importantly, in the Morelli study, Fas ligand (FasL) transgene expression and induced-toxicity following IV delivery were restricted to glial tissue, unlike the noncell-type-specific CMV-driven viruses that induced acute liver haemorrhage with hepatocyte apoptosis. Tight control of transgene expression was therefore achieved following systemic delivery. In the Chen study, a repressible adenovirus was developed by driving expression of a tetracycline-controlled transactivator. This was the first glial-specific gene delivery system that allowed for repression of ectopic gene expression. More recently, an elegant liposomal delivery system has been developed; it incorporates both transport across the blood brain barrier following IV administration of a targeting ligand such as the peptidomimetic rat 8D3 mAb to the mouse transferrin

receptor, and restricted expression to glial cells by the use of the GFAP promoter. This particular study demonstrates that tissue-specific gene expression in brain is possible after IV administration of a nonviral vector combined with gene targeting technology and glial-specific promoters [50]. Shinoura et al [52, 53, 54] have used the MBP promoter to drive expression of two apoptosis-related genes, *BAX* and caspase 8, selectively, in glioma cells both *in vitro*, and *in vivo* using adenoviruses. Massive apoptosis was seen in glioma cells only, demonstrating effective tissue specificity and good antitumour efficacy was observed *in vivo*.

As well as promoters that can target gliomas, a promoter that could be useful in targeting neuroblastomas has also been described. The human NCX gene, is a homologue of the murine neural crest homeobox (*Ncx/Hox11L.1*) gene, whose expression is restricted to a subset of neural crest-derived tissues. A 1.7-kbp fragment upstream showed preferential promoter activity in neuroblastoma cells and when linked to the HSV-*tk* gene caused increased sensitivity to gancyclovir [59]. The regulatory region of this gene may therefore be useful for neuroblastoma-specific gene therapy.

#### **Liver-specific expression**

The enhancer/promoter of the albumin gene is the only tissue-specific promoter that has been shown to preferentially target liver cells. The first demonstration of efficacy using this promoter was by Kuriyama et al [60]. When this promoter was delivered using a retrovirus, which is efficient only in dividing cells,  $\beta$ -galactosidase expression was restricted to hepatoma cell lines *in vitro*. When this retrovirus was injected via the spleen or directly into the liver, gene expression was observed only in dividing hepatocytes in partially hepatectomised mice, but not in nondividing hepatocytes in normal mice. These authors went on to show that the susceptibility of murine and rat hepatocellular carcinoma (HCC) cells, infected with retroviruses expressing HSV-*tk* under the control of the albumin promoter, were 100-fold more sensitive to gancyclovir than non-HCC cells. Systemic gancyclovir administration resulted in complete regression of retroviral-infected HCC cells and significant inhibition of tumour growth even when only 5% of the cells were infected with retrovirus [61]. The albumin promoter has also been used to develop a retrovirus expressing the TNF- $\alpha$ , IL-2, and IL-3 genes [62, 63], an adenovirus expressing HSV-*tk* [64, 65] and a replication-competent herpes simplex virus, HSV G92A [66].

#### **Thyroid-specific promoters**

Thyroid cancer is a disease with a relatively good prognosis, but about 30% of the tumours dedifferentiate and may finally develop into highly malignant anaplastic thyroid carcinomas which are inaccessible to standard therapeutic procedures such as radioiodine therapy and thyroxine-mediated thyrotrophin suppression. Several

gene therapy strategies are now available to target therapeutic genes to thyroid tissues using tissue-specific promoters. Zeiger first described the use of the thyroglobulin (TG) gene promoter for the selective targeting of thyroid cell lines. Nearly, 100% of thyroid carcinoma cells treated with an adenovirus expressing HSV-*tk* under the control of a TG promoter were killed by GCV compared to only 5% of control cells [69]. A retrovirus carrying HSV-*tk* under the control of the TG promoter also showed preferential GCV toxicity for malignant rat thyroid carcinoma cells compared to a normal rat thyroid cell line *in vitro* and *in vivo* [70]. Furthermore, systemic delivery of an adenovirus expressing thyroglobulin-driven HSV-*tk* did not induce luciferase activity in the liver or spleen, indicating that TG does not induce luciferase expression in non-TG producing tissues [71]. In addition, transfer of the HSV-*tk* gene driven by the TG promoter following administration of GCV is specifically cytotoxic to thyroid cells [71]. In order to address the issue that most poorly differentiated and anaplastic thyroid carcinomas have lost the ability to express the TG gene through loss of the thyroid transcription factor-1 (TTF-1), TTF-2, or Pax 8, Shimura et al infected thyroid cells with both adenoviruses expressing TTF-1 and adenoviruses carrying HSV-*tk* driven by the thyroglobulin promoter [72]. Cotransduction permitted 90% cytotoxicity in thyroid carcinoma cells lacking this transcription factor and normally unable to express the TG gene. A putative enhancer element for the TG gene has also been recently identified which appears to increase the TG promoter activity and specificity for thyroid cells. Interestingly, the TG promoter is also being used to target feline thyroid disease and neoplasia by selectively increasing expression of the nitroreductase gene allowing subsequent activation of a prodrug, CB1954 [73].

Several other novel tissue-specific genes have been used to target tumours in a tissue-specific manner, although only one or two reports are available. These include an ovarian-specific promoter, OSP-1, a 462-bp sequence that is a part of a retrovirus-like element specifically expressed in rat ovary [74]. The expression of HSV-*tk* under the control of this promoter significantly sensitised ovarian cancer xenografts (OVCAR) to GCV following delivery with a cationic lipid [75]. In addition, the promoter for surfactant protein B (SPB), whose activity is restricted to adult type II alveolar epithelial cells and bronchial epithelial cells, has been used to target both replication-deficient [67] and -competent adenoviruses by replacement of the E4 promoter [68]. This promoter showed good specificity for bronchial cancer cells *in vivo*.

#### **TUMOUR-SPECIFIC PROMOTERS**

Ideally, tumour-specific promoters should be highly active in tumour cells and have little or no activity in normal cells. Harrington et al [78] elegantly split this vast group of promoters into subgroups depending on

their discrete characteristics and for the purpose of this review this convention will be applied. Tumour-specific promoters can be described as (1) cancer-specific promoters, that is, specific for the malignant process, but showing no tissue specificity (2) tumour-type-specific promoters, that is, oncofoetal related with tissue specificity (3) tumour microenvironment-related promoters, that is, promoters which respond to the tumour microenvironment and physiology, for example, hypoxia and glucose regulated (4) tumour vasculature-related promoters, that is, promoters which are more active in the tumour vasculature rather than normal vasculature.

### Cancer-specific promoters

One of the main obstacles to current cancer therapies is the lack of tumour specificity. Targeting gene expression specifically to tumour cells is therefore one of the most important goals of cancer gene therapy strategies. A common problem with the use of the tissue-specific promoters which were described in the previous section, is that transgene expression can also occur in the normal tissue and whilst some tissue damage may be acceptable in certain organs, in other organs such as brain, liver, and so forth, it could be catastrophic. There is probably only one gene, telomerase, that can be genuinely classified as cancer specific and whose promoter is being used to drive the expression of transgenes selectively in a wide variety of tumour cells.

Telomerase activation is considered to be a critical step in cancer progression because of its role in cellular immortalisation. Approximately 90% of human cancers possess active telomerase, whereas normal somatic tissues have either much lower or undetectable activity [79, 80, 81]. Human telomerase activity depends on the presence of both the RNA subunit (hTR) and the catalytic protein component (hTERT) [82]. For both genes there is a clear differential expression between normal and malignant cells. A number of studies have used these two promoters to drive transgene expression for therapeutic benefit. Pan and Koeneman [83] first described the use of the hTERT promoter in a retroviral system and combined it with a *Cre/loxP* site-specific recombination system to allow the killing of p53-negative tumour cells while sparing normal wild-type cells. Since then, a host of papers have reported the use of both promoters in several different tumour types in combination with several different transgenes. Bladder and liver tumour cell lines were targeted using both hTR and hTERT to drive expression of the diphtheria toxin A chain gene [84]. The glioma cell line, UVW, was targeted using the hTR promoter to drive expression of the noradrenaline transporter gene (NAT) where a 17-fold enhancement of the radionuclide [<sup>131</sup>I]meta-iodobenzylguanidine uptake was reported resulting in complete sterilisation of tumour spheroids [85]. Gliomas were also the target for hTERT-driven expression of Fas-associated protein with death domain (FADD) or rev-caspase-6. FADD is a protein which induces apoptosis

in cells regardless of Fas expression on the cell surface and rev-caspase 6 induces apoptosis independent of initiator caspases. Subcutaneously implanted human gliomas treated with this construct were significantly reduced in volume compared to control tumours [86, 87, 88]. More recently, the apoptotic pathway was successfully targeted both in human tumour xenograft and syngeneic mouse UV-2237m fibrosarcomas, using Bax as a transgene, driven by the hTERT promoter [89, 90]. In a suicide gene therapy approach, hTERT was also used to drive expression of HSV-tk in an osteosarcoma xenograft model following intratumoural injection. Targeted delivery using this approach avoided the liver toxicity observed using a CMV driven construct following GCV administration. Finally, Keith's group [91] tested hTR and hTERT in a panel of 10 cell lines consisting of lung, colon, ovarian, bladder, and cervical origin, demonstrating the truly "cancer specific" nature of these promoters. The hTR promoter appeared to have the superior activity in the telomerase-positive cell lines and when used to drive the nitroreductase gene to increase the activation of the pro-drug, CB1954, in two xenograft models, it showed even better antitumour effects (up to 97% reduction in tumour volume) than a CMV-driven construct [91]. These promoters clearly have a real potential in targeting a wide range of different tumour types.

### Tumour-type-specific promoters

Certain types of tumour often overexpress genes of oncofoetal origin that are silent in normal tissue. The most well-characterised promoters of these tumour-specific genes are the carcinoembryonic antigen (CEA) and  $\alpha$  fetoprotein (AFP), which are expressed in adenocarcinomas and hepatocellular carcinomas, respectively. There are also many other genes that are overexpressed in certain tumour types and, as such, their promoters are being utilised for transcriptional regulation of therapeutic genes.

#### CEA

The CEA promoter is active in a proportion of breast, lung, colorectal, and pancreatic cancers. The 5' promoter sequences have been analysed and identified by reporter gene assays and have lead to high-level selective expression of transgenes in CEA-positive cell lines [92]. The CEA promoter has been used to drive CD expression [92, 93, 94], HSV-tk [95, 96, 97, 98, 99], BAX [100], and mammalian degenerin, a sodium ion channel gene, overexpression of which leads to sodium influx and cell bursting [101]. Importantly, CEA appears to be truly selective for tumour tissues in animal models as several reports have not detected transgene expression or related toxicities following systemic delivery of these constructs via tail vein injection [98] or intraperitoneally [94]. Although a significant number of studies have shown that the CEA promoter is effective for selectively targeting tumour cells, there have been few studies which have demonstrated

truly significant *in vivo* antitumour effects possibly because of the low activity of the promoter. More recently, several studies have made use of a Cre recombinase/*loxP* system [102, 103, 104] or a GAL4 regulatory system [100], both of which significantly increased transgene expression by up to 100 fold, while maintaining tumour specificity. These strategies will be discussed in more detail later. Finally, Qiao et al [105] constructed an adenovirus containing a binary promoter system with a CEA promoter driving a transcription activator, which then activates a minimal promoter to express HSV-*tk*. Importantly, *in vivo* dose-escalation studies demonstrated significantly reduced toxicity following intravenous administration of this construct and provide a proof of principle for the powerful enhancement of a weak promoter for effective tumour-restricted gene expression.

#### AFP

The  $\alpha$ -fetoprotein gene is normally expressed in foetal liver and is transcriptionally silent in adult liver. It is also expressed in around 70% of the hepatocellular carcinomas and its promoter has been used extensively to drive transgene expression for targeting these tumours. Studies have clearly shown that transgene expression is restricted to tumours that overexpress AFP and that transgene expression is less efficient in tumours with lower AFP levels. A variant 0.3-kbp promoter has also been developed with a G to A substitution at nucleotide -119; a point mutation responsible for hereditary persistence of human AFP. This promoter has proved particularly useful for transgene expression in low AFP producing hepatoma cells [106]. Ishikawa et al have also shown that by placing the AFP promoter/enhancer in the reverse orientation to the long terminal repeats with a retroviral construct dramatically increases both HSV-*tk* transgene expression and GCV-mediated cytotoxicity in a HepG2 tumour *in vivo* [107]. The AFP promoter has also been used to drive CD [108], IL-2 [109, 110], diphtheria toxin A [111], and interestingly, a replication-competent adenovirus has been constructed using the AFP promoter to drive an attenuated E1B gene. Systemic delivery of this virus resulted in tumour-specific regression [112]. The AFP promoter activity can be weak, but it can be enhanced using Cre/*loxP* technology to increase transgene expression [113], a similar strategy to that applied to CEA.

#### ErbB2

ErbB2/HER2 is an oncogene that codes for a receptor tyrosine kinase that is overexpressed by transcriptional upregulation in approximately one third of breast and pancreatic tumours, and in a small proportion of other tumours. This promoter has been used to drive the expression of CD and when delivered using a retrovirus, CD expression and significant 5-FC-mediated cell death was observed only in ErbB2-positive cell lines [114, 115]. ErbB2-driven expression of a membrane receptor, murine

gastrin releasing peptide, has also been used effectively to target breast cancer cell lines with a radiolabelled peptide [116]. More recently, studies to identify a minimal ErbB2 promoter were successful. Maeda et al [117] identified a 251 bp fragment (-213/+38) that could direct transcription of a luciferase reporter gene better than the SV40 immediate early promoter in breast cancer cells. When linked to the HSV-*tk* gene, increased sensitivity to GCV was observed in breast but not nonbreast cancer cell lines, and suppressed the growth of these lines in nude mice.

#### MUC1/DF3

The MUC1 gene encodes a high molecular weight mucin-like glycoprotein and is overexpressed at the transcriptional level in breast and cholangiocarcinomas. A 114 bp enhancer region has been identified that can modulate transcription from heterologous promoters [118]. DF3-positive breast cancer cell lines were more sensitive to GCV-mediated cell killing when the HSV-*tk* gene was delivered and driven by this enhancer. A replication-defective adenovirus containing this construct was subsequently developed and inhibition of tumour growth was observed in an intraperitoneal breast cancer metastases model [119]. This strategy could transfect and kill carcinoma cells within haematopoietic stem cell populations without causing associated normal tissue toxicity [120]. An adenovirus expressing BAX under the control of this promoter has also been constructed for targeting ovarian cancer cell lines expressing DF3. Transgene expression was restricted to tumour tissue in a nude mouse model and administration of the virus 2–3 days after tumour inoculation eradicated > 99% of tumour implants [121].

#### Osteocalcin

Osteocalcin (OC) is a noncollagenous bone matrix protein expressed at high levels in the neonate. It functions primarily in osteoblasts found in growing bone and is highly expressed in osteogenic sarcomas. Koeneman et al [122] have also provided evidence of expression in ovarian, lung, brain, and prostate tumours (especially those showing androgen-independence). A phase I trial is now underway to target androgen-independent prostate cancer using an adenoviral construct expressing HSV-*tk* under the control of this promoter. Of all the transcriptional targeting strategies discussed so far, the use of the osteocalcin promoter has been tested thoroughly using systemic delivery. Matsubara et al [123] have successfully constructed a replication-competent adenovirus using this promoter to drive Ela expression. They demonstrated that 100% of mice carrying prostate xenografts responded to systemic adenoviral delivery and more significantly 40% of mice were cured. Phase I/II trials are now being carried out using the same virus to target pulmonary metastases of osteosarcomas, following systemic delivery of this virus, which must pass through the lung before being sequestered in the liver [124].

Shirakawa et al [125] also used an adenovirus to target osteosarcoma pulmonary metastases with an osteocalcin driven HSV-*tk*. In an established animal model, systemic delivery of these constructs via tail vein injection and subsequent intraperitoneal acyclovir treatment significantly decreased the number of tumour nodules in the lung, while significantly increasing the survival of these animals. Interestingly, because the osteocalcin promoter can also target bone stromal cells, which are important for supporting the growth of prostate tumour metastases, an adenovirus expressing HSV-*tk* under the control of this promoter was effective at destroying prostate cancer xenografts in both subcutaneous and bone sites [122]. It is quite clear that this promoter will be important for the treatment of both prostate and osteosarcoma, but it will be some time before we learn about its true specificity and any associated toxicities. Certainly, because this promoter is effective in androgen-independent prostate tumours, it will ultimately be more effective than the tissue-specific PSA promoter discussed in the last section. A clearer understanding of this promoter may help to increase specificity even further and Yeung et al have already identified elements important for prostate specificity, in particular, the elements important for androgen-independent specificity [126].

#### **L-plastin**

L-plastin is constitutively expressed at high levels in malignant epithelial cells, but is not expressed in normal tissue, except at low levels in haemopoietic cells. A replication-incompetent adenovirus has been constructed to express either LacZ or CD under the control of the human L-plastin promoter. High levels of expression of  $\beta$ -galactosidase was seen in breast, ovarian, and fibrosarcoma cell lines [127] and in explants of ovarian cancer cells from patients compared to normal mesothelial cells from surgical specimens [128]. A greater reduction in tumour size was seen when the adenovirus expressing CD under the control of the L-plastin promoter was injected into human ovarian tumour xenografts than when the CD was controlled by the CMV promoter. L-plastin may therefore be an effective promoter for targeting ovarian cancer.

#### **Midkine**

Midkine (MK) is a heparin-binding growth factor that is transiently expressed in the early stages of retinoic acid-induced differentiation of embryonal carcinoma cells. Many malignant tumours express high levels of MK mRNA or protein with no expression in human liver. This is important as adenovirus' have a natural tropism for liver and such promoters could be useful for preventing transgene expression and associated hepatotoxicity in the liver. Miyauchi et al [129] examined the expression of MK in human oesophageal cancer cells. Positive staining with anti-MK antibody was found in 8 out

of 14 tumour specimens, while the surrounding normal oesophageal tissues in these specimens were not stained. The 5' flanking 2.3-kbp region of the MK gene was then used to drive the expression of HSV-*tk*, where expression conferred the sensitivity to GCV. Since then, an adenoviral vector has been constructed to express HSV-*tk* under the control of the MK promoter to target Wilm's tumour and neuroblastoma cell lines [130]. Importantly, systemic delivery of these constructs into mice resulted in much lower hepatotoxicities; by day 8 of systemic delivery, four out of five mice treated with an adenovirus expressing HSV-*tk* via a CMV promoter had died, whereas all the mice treated with the midkine adenovirus survived at least 10 days [131]. The midkine promoter has also proved useful for targeting ovarian cancer cells and pancreatic cells, as has the cyclooxygenase-2 gene promoter [132, 133].

#### **Other tumour-type-specific promoters**

Many other tumour-specific promoters have been utilised to target particular types and are listed in Table 2. Particularly interesting is the secretory leukoprotease inhibitor gene (SPL1) promoter for targeting cervical carcinoma cells [157], lung, breast oropharyngeal, bladder, endometrial, and colorectal carcinomas [158]. Yamamura et al [170] have used the promoter from the human calponin gene, normally expressed in matured smooth muscle cells and overexpressed in human soft tissue and bone tumour cells, to drive expression of ICP4, a major transacting factor for viral genes to produce a novel conditionally replicating herpes simplex virus. The viral treatment showed stable and significant inhibition of tumourigenicity with cures in four out of five mice harbouring synovial sarcoma, leimyosarcoma, and osteosarcoma cells, while sparing normal vascular smooth muscle cells. Chen and McCormick [172] report on a novel transcriptional targeting strategy for colon cancer. Since many colon cancer cells show mutations in either the adenomatous polyposis coli or  $\beta$ -catenin genes that lead to stabilisation of  $\beta$ -catenin and activation of downstream T-cell factor (Tcf) target genes, tcf promoter elements were used to control expression of an apoptosis gene, FADD, which appears to effectively and selectively kill colon cancer cells. The regulatory regions of the H19 gene, is differentially regulated in normal and cancer cells and has been linked to diphtheria toxin A or HSV-*tk* to successfully target syngeneic bladder tumours in vivo with no obvious systemic toxicity towards the host animals [171]. A 2.2-kbp 5' flanking region of the human calretinin gene has been used to drive the expression of HSV-*tk* selectively in mesothelioma cell lines [174] and the human calcitonin promoter has been used to drive expression of HSV-*tk* in medullary thyroid carcinomas following systemic delivery by adenovirus without any evident toxicity [175, 176, 177]. The gastrin-releasing peptide gene promoter has been used to selectively control HSV-*tk* expression in small-cell lung cancer cells [167]; injection of an adenovirus containing this construct resulted in complete regression of lung

TABLE 2. Tumour-specific promoters.

Promoter	Tumour target	Reference
Telomerase	Lung, colon, ovarian, bladder, cervical, liver, glioma	[83, 84, 85, 86, 87, 91, 134, 135, 136, 137]
CEA	Colorectal, pancreatic, cholangiocarcinoma, breast, lung	[92, 93, 94, 95, 96, 97, 98, 100, 138, 139, 140, 141, 142, 143, 144]
Alpha feto protein (AFP)	Hepatoma	[64, 106, 107, 108, 145, 146, 147, 148, 149]
Erb B2	Breast, pancreatic, ovarian	[114, 115, 116, 117, 150, 151, 152]
DF3/MUC1	Breast, cholangiocarcinoma	[116, 118, 119, 120, 121, 153, 154]
Osteocalcin	Prostate, ovary, lung, brain, osteoblasts	[122, 123, 124, 125, 126, 127, 155, 156]
L-plastin	Ovarian, breast, fibrosarcoma	[127, 128]
Midkine	Embryonal carcinoma; Wilm's tumours, neuroblastoma, pancreatic, oesophageal	[129, 130, 131, 132, 133]
Secretory leukoprotease inhibitor (SLP1)	Lung, breast, oropharyngeal, bladder, endometrial, ovarian, colorectal, cervical	[157, 158]
Alpha lactalbumin	Breast	[159]
Myc-max	Breast, lung	[140, 160, 161]
Somatostatin	Malignant melanoma of soft parts	[162]
Cox2	Ovarian, pancreatic, gastroin-testinal	[132, 133, 163]
Ornithine decarboxylase	Colon and neuroblastoma	[164]
Epithelial glycoprotein 2 (EPG2)	Carcinomas	[165]
c-Myb-responsive promoters	Hematopoietic tumours	[166]
Gastrin-releasing peptide	Lung	[167, 168]
Metallothionein	Ovarian	[169]
Calponin	Soft tissue and bone tumours	[170]
H19	Bladder	[171]
Tcf	Colon	[172, 173]
Calretinin	Mesothelioma	[174]
Calcitonin/calcitonin gene-related peptide	Thyroid/thyroid medullary cancer	[175, 176, 177]
<b>Cell cycle-related</b>		
Cdc25C	Melanoma	[178]
CyclinA	Melanoma	[13, 178, 179]
Endoglin	Endothelial cells	[179, 180]
IGF-1-R	Tumours mutant for p53, cMyb or EWS/WT1	[181]
E2F-1	Glioma	[182]

tumour xenografts following IP administration of GCV [168]. Lastly, Vandier et al [169] have been successful in targeting cisplatin-resistant ovarian carcinoma cells which overexpress the metallothionein gene, a metal binding protein. The human metallothionein promoter was therefore used to drive expression of HSV-tk, resulting in marked sensitisation to GCV in the cisplatin-resistant cell lines compared to the cisplatin sensitive cells.

#### **Cell cycle-related promoters**

The expression of proliferation-associated genes is a hallmark of both cancer cells and tumour endothelial

cells. The retinoblastoma tumour suppressor gene (RB), is a cell cycle-associated gene, which has the ability to repress E2F-responsive promoters, such as E2F-1. E2F-responsive promoters should therefore be more active in tumour cells relative to normal cells because of an excess of free E2F and loss of RB/E2F repressor complexes. Parr et al [182] have shown that adenoviral vectors, containing transgenes driven by an E2F-1 promoter, can mediate tumour-selective gene expression *in vivo*, allowing for eradication of established tumours. Significantly, less toxicity was seen with this adenovirus than standard constitutively regulated adenoviral constructs.

TABLE 3. Tumour environment-specific promoters.

Promoter	Tumour target	Reference
<b>Endothelial cell-specific promoters</b>		
KDR/flk-1	Sarcoma, ovarian, endothelioma	[184, 185, 186, 187]
Flt-1	Breast, gynaecological, teratocarcinoma	[188]
E-selectin	Endothelioma	[184, 186, 189]
von Willebrand factor (vWF)	Endothelial cells	[190]
Preproendothelin-1	Endothelial cells	[191, 192, 193]
VCAM-1	Endothelial cells	[186]
<b>Hypoxia-responsive promoters</b>		
VEGF (HRE elements)	Hepatoma	[145, 194, 195, 196, 197]
Erythropoietin (HRE elements)	Brain	[198]
Phosphoglycerate kinase 1 (HRE elements)	Fibrosarcoma	[33, 199]
<b>Glucose-responsive promoters</b>		
GRP78	Breast	[198, 200, 201, 202, 203, 204, 205]
Hexokinase II	Lung	[206]

Muller's group have published several papers where the cell cycle-related promoter, cyclin A or cdc25c, has been used in combination with a tissue-specific tyrosinase promoter to selectively target proliferating melanoma cells [13, 178]. These dual specificity promoters showed > 20 fold and > 50 fold increase in cell cycle-related and cell-type-specific regulation, respectively, and will be discussed in more detail later.

Finally, the insulin-like growth factor-1 (IGF-1) has a central role in normal cellular proliferation. Transcription of this gene is controlled by a number of tumour suppressors. On the other hand, a number of oncogenes, including mutant *p53* and *c-Myb* and the fusion protein EWS-WT1 significantly stimulate promoter activity. This promoter may therefore be a good candidate for use in transcriptional targeting in cancer gene therapy protocols [181].

#### **Promoters that respond to the tumour microenvironment and physiology**

There are many aspects of the tumour microenvironment that are unique, and can be targeted with strategies that take advantage of genes upregulated in response to these environments. For tumours to grow, they must stimulate angiogenesis; this requires proliferation of endothelial cells, a process specific (in normal adults) to the tumour microenvironment. An additional bonus of this strategy is that vectors delivered systemically will have direct access to the proliferating endothelial cells of the tumour vasculature. Furthermore, specific genes are upregulated in proliferating endothelial cells whose promoters are attractive for targeting transgenes to the tumour vasculature. Secondly, because the tumour vasculature is often disorganised and inadequate, areas of low oxygen tension (hypoxia) are prevalent in malignant tumours [183]. Many genes transcriptionally upregulated in response to

hypoxia are mediated by the inducible transcription complex, hypoxia-inducible factor-1 (HIF-1). HIF-1 binds to DNA motifs known as hypoxia response elements (HREs) within these genes. HREs can therefore be used to drive transgene expression specifically within areas of tumour hypoxia. It is extremely important to target this population of cells since they are highly resistant to other forms of treatment, such as radio and chemotherapy. Lastly, as well as oxygen starvation, tumours can also be deprived of glucose leading to the increased expression of genes involved in glucose metabolism. The promoters of these genes are therefore also being used to drive transgene expression specifically within a tumour. A list of tumour microenvironment and physiology-related promoters can be found in Table 3.

#### **Endothelial cell-specific promoters**

Over the past 10 years a number of genes have been reported to be upregulated in proliferating endothelium of tumour blood vessels. The endothelial-specific kinase insert domain receptor (KDR/flk-1) and E-selectin have been shown to be upregulated in tumour endothelium, and defined regions of these promoters have been used to drive TNF- $\alpha$  carried by a retroviral vector. A 10–11-fold increase in TNF- $\alpha$  expression within sEND endothelioma cells was observed compared to NIH-3T3 fibroblasts [184]. More recently, it was demonstrated that the KDR/flk-1 promoter, is not only endothelial cell-specific, but also active in two human ovarian cancer cell lines where CD expression sensitised these cells to 5-FC both in vitro and in vivo [185]. E-selectin expression is very low in normal adult capillaries, but is significantly elevated in newly formed tumour vessels. It has been shown that the E-selectin promoter is also activated by TNF- $\alpha$ , generating a 30-fold increase in transgene expression compared

to untreated cells. Furthermore, endothelial cells exposed to tumour-conditioned medium as an in vitro simulation of the tumour microenvironment resulted in even higher induction of transgene expression [189].

Flt-1, a receptor for vascular endothelial growth factor (VEGF), is known to display dysregulated expression in both tumour vasculature and tumour cells per se, and adenoviral vectors containing transgenes under the control of the flt-1 promoter achieve very low levels of toxicity in normal liver. This is important, as this is the major organ responsible for blood clearance of adenoviruses and most at risk from inadvertent transgene-related toxicity. Bauerschmitz et al [188] have shown that the flt-1 promoter is useful for the transcriptional targeting of adenoviruses to teratocarcinomas.

The promoter of human preproendothelin-1 has been used to generate a retrovirus that demonstrates endothelial-specific expression of the  $\beta$ -galactosidase gene [191, 192]. An adenovirus containing the preproendothelin-1 promoter driving luciferase expression has also been constructed. Following systemic injection, higher transgene expression was seen using this promoter rather than a CMV promoter, resulting in a high level of activity specifically in the new vasculature of primary tumours and lung metastases, with the highest level in the angiogenic endothelial cells of the metastasis [193]. However, an impediment to the use of the preproendothelin-1 promoter may be that it is also active in some epithelial cells [207], compromising its vascular targeting specificity.

The promoter for human von Willebrand factor (vWF) has also been used to drive transgene expression selectively in proliferating endothelial cells [190, 208]. Both reports emphasise that although this promoter is very weak, it appears to be highly selective for endothelial cells and is effective at suppressing endothelial cell growth when driving the HSV-tk gene in the presence of GCV. Furthermore, a novel strategy has been developed to enhance this promoter > 14–100 fold while maintaining > 30–100 fold specificity and will be discussed in more detail later [208]. Nettelbeck also tested the PECAM-1, endoglin, P-selectin, and KDR promoters, but vWF promoter was clearly the most specific for endothelial cells.

#### Hypoxia-responsive promoters

Hypoxia response elements (HREs) found within the hypoxia-responsive genes of vascular endothelial growth factor (VEGF), erythropoietin (Epo), and phosphoglycerate kinase-1 (PGK-1), have been tested for their use in cancer gene therapy. Shibata et al examined the HREs of human VEGF and Epo and found high inducibility of both in mammalian cells, while only the HREs from the VEGF promoter were active in murine cells, and then to a lesser extent than in human cells. In addition, the introduction of multiple copies of the HRE and an E1B minimal promoter improved the hypoxia responsiveness [194]. Shibata then tested other HRE promoter combinations and found that 5HREs and a CMV minimal

promoter exhibited the most effective hypoxia response (over 500 fold) when compared to an intact CMV promoter [195]. In addition, significant antitumour effects were achieved with intraperitoneal injections of the prodrug, CB1954, in tumours that expressed bacterial nitroreductase (NTR) constitutively or via this hypoxia-inducible promoter. Furthermore, respiration of 10% O<sub>2</sub> increased tumour hypoxia in vivo and enhanced the antitumour effects [196]. The HREs of the mouse VEGF gene have also been used to construct a retroviral vector expressing HSV-tk and tumours expressing this construct regressed following administration of GCV [197]. Dachs et al [199] have successfully utilised HREs from the PGK-1 gene and have shown that it is active in hypoxic tumour cells in vivo and Ruan et al [198] have constructed an adeno-associated virus carrying HREs from the Epo gene that can increase gene expression in brain tumour cell lines, under anoxia, by 79–110 fold. Combinations of both hypoxia and tumour/tissue-specific elements are now being developed to increase specificity for hypoxic tumour cells [145, 150, 186, 209]. These will be discussed in more detail later.

#### Glucose-responsive promoters

The glucose-regulated protein GRP78 functions as a molecular chaperone and can bind to malfolded proteins and unassembled complexes. They are induced in response to low glucose, hypoxia, acidic pH, or oxidative stress, which are hallmark features of solid tumours. The use of these promoters for cancer gene therapy was first proposed by Lee's group [200]. This group has fully characterised the use of this promoter in several tumour systems. A truncated rat GRP78 promoter with most of its distal basal elements removed drove high level expression in a murine fibrosarcoma model [201] and when GRP78 was used in a retroviral system to drive HSV-tk, HSV-tk expression levels were much higher [202] in tumours in vivo than cells in vitro, resulting in complete tumour eradication with no recurrence of tumour growth. Similarly, impressive results were obtained in a murine mammary adenocarcinoma cell line (TSA) in syngeneic, immune-competent hosts [203]; in addition, immune memory was induced in these hosts with this system. Finally, Luna et al [204] have used photodynamic therapy (PDT) to activate the GRP78 promoter, since this modality generates cytotoxic singlet oxygen species, which are potent activators of this promoter. Inducible expression of HSV-tk was observed after PDT in stably retrovirally-transduced mouse mammary carcinoma cells (TSA), and enhanced tumourcidal activity was seen. The GRP78 promoter is, therefore, also inducible and can function as a molecular switch following exposure to PDT.

Hexokinase II is also overexpressed in solid tumours, partly due to elevated glucose catabolism in rapidly proliferating cells. Hexokinase II catalyses the first committed step of glycolysis and is overexpressed in some tumours since it is no longer responsive to the physiological

TABLE 4. Exogenously controlled inducible promoters.

Promoter	Tumour target	Reference
<b>Radiation-inducible promoters</b>		
Egr-1 and CArG elements	Glioma, hepatocellular carcinoma, soft tissue sarcomas	[209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221]
Waf-1	Murine fibrosarcoma	[222, 223]
RecA	Clostridia	[224, 225]
c-IAP2	Colon	[226]
<b>Heat-inducible promoters</b>		
HSP70B	Breast, melanoma, and prostate	[227, 228, 229, 230, 231, 232, 233, 234]
Gadd 153		[235]
<b>Drug-inducible promoters</b>		
MDR-1	Breast, colon	[236, 237, 238]
Tetracycline inducible	Breast, melanoma, brain, glioma, prostate	[49, 146, 239, 240, 241, 242, 243, 244, 245, 246, 247]
Rapamycin inducible	Fibrosarcomas	[248]
Tamoxifen-inducible estrogen response elements	Breast	[150, 249, 250]

inhibitor, glucagon. Adenovirus carrying *LacZ* under the control of the hexokinase promoter showed preferential  $\beta$ -galactosidase expression in non-small cell lung carcinomas compared to primary normal human bronchial epithelial cells. Cells infected with an adenovirus expressing HSV-*tk* under the control of this promoter, demonstrated selective toxicity, with a 10 to 100-fold increase in IC<sub>50</sub> between the lung cancer cell lines and the normal bronchial epithelial cells and a 100-fold increase in the IC<sub>50</sub> when compared to MCF-7 breast carcinoma cells [206].

#### EXOGENOUSLY CONTROLLED INDUCIBLE PROMOTERS

The use of tissue/tumour-specific cellular promoters to restrict transgene expression usually results in constitutive expression in the target tissue. However, in many situations it might be preferable to exogenously regulate the duration and level of expression. A list of exogenously controlled inducible promoters currently being used for cancer gene therapy purposes can be found in Table 4.

##### Radiation-inducible promoters

Radiation therapy is one of the main strategies used to treat solid tumours and although successful in some cases, there is the potential for enhancing radiation effects by combining with other agents such as gene therapy. Radiation-mediated gene therapy exploits the fact that as well as the cell killing effect, radiation will also activate transgenes which are driven by a radiation-inducible promoter. Confinement of transcription to the radiation field should be achievable by either using conformal, interstitial, or brachytherapy and by targeted delivery of radioisotopes. The cellular response to ionising radiation includes

the transcriptional activation of a variety of genes, for example, *c-jun*, *NFB*, *EGR1*, and *p21<sup>WAF1/CIP1</sup>*, to name but a few. However, although numerous radiation-inducible genes and proteins have been identified, relatively few radiation-inducible promoters/enhancers have been fully characterised.

Datta et al [251] identified a sequence of the EGR-1 gene that confers radiation inducibility. This sequence, CC(A+T rich)GG, is known as the radiation-responsive CArG element. In vitro studies, using the CArG, elements of the EGR1 promoter have demonstrated a dose-dependent expression of reporter gene activity of up to 28 fold following radiation exposure (0–20 Gy) [210]. However, at therapeutically relevant doses of radiation (2 Gy) only a 3-fold increase in reporter gene activity was achievable [211]. The thymidine analogue 5-iodo-2'-deoxyuridine radiolabelled with the Auger electron-emitter iodine-125 also activated the promoter and resulted in increased  $\beta$ -galactosidase activity in the 9L gliosarcoma tumour model in vivo. A number of studies have used CArG elements to drive expression of TNF using liposomes [212], a haemopoietic cell carrier [213] and adenovirus [214, 215, 216]. These studies have led to clinical trials where an adenovirus carrying TNF driven by CArG elements of the Egr-1 promoter are injected intratumourally into patients with soft-tissue sarcomas, followed by irradiation [217]. Enhanced antitumour effects were also observed when the Egr-1 promoter was combined with HSV-*tk* [210, 252]. Tumours regressed significantly after GCV and radiation in comparison to GCV alone. Furthermore, tumour regrowth did not occur when GCV treatment was withdrawn. As well as being radiation-inducible the Egr-1 promoter was tumour-specific for hepatocellular carcinomas whereas its expression was barely detectable within normal liver [210]. It

was evident from some of these papers that reporter gene expression was detectable in nonirradiated cells, which could limit its use in combination with cytotoxic agents. Weichselbaum et al [218] are also developing a herpes simplex virus (HSV-1) that is induced to proliferate within the tumour volume following ionising radiation exposure. Finally, Marples and Scott are developing a novel molecular switch using CArG elements derived from this promoter, in combination with a Cre/loxP site-specific recombination system of the P1 bacteriophage, to generate high level and constitutive expression of transgenes following activation by therapeutically relevant radiation doses [209, 219, 253]. This system will be discussed in more detail later.

The promoter of the P21<sup>WAF1/CIP1</sup> gene is also radiation-sensitive and its activation is also increased in tumour cells [254, 255, 256, 257, 258, 259, 260, 261]. Interestingly, expression appears to be independent of p53 status in a variety of tumour types [257, 262, 263]. Furthermore, we have evidence that this promoter is also highly active following exposure of cell in vitro to hypoxia (unpublished data, 2002). This promoter therefore, may have several layers of specificity, that is, tumour-, radiation-, and hypoxia-specific. Research in our laboratory has shown that WAF-1 can successfully drive expression of inducible nitric oxide synthase which leads to the generation of the vasoactive, cytotoxic, and radiosensitizing product, nitric oxide (NO), close to therapeutically relevant doses. As well as causing significant vasorelaxation in isolated arteries [222], it also caused abundant apoptosis in a murine fibrosarcoma model and a highly significant tumour growth delay in vivo when used in combination with 20 Gy radiation [223]. This promoter is also being used to drive expression of cytochrome P450 enzymes to increase the activation of the bioreductive drug, AQ4N [264, 265].

Radiation-inducible bacterial promoters have also been used such as RecA, delivered using the anaerobic apathogenic bacterium, clostridia, as a gene transfer system to target anoxic areas within tumours. Significant increases in  $\beta$ -galactosidase activity and TNF- $\alpha$  secretion was seen following 2-Gy irradiation [224, 225, 266]; fractionation of the radiation dose lead to repeated gene induction.

Lastly, a retrovirus gene trap screening identified c-IAP2 as an X-ray-inducible gene with radiation-sensitive NFkB binding sites. Construction of a plasmid with four tandem repeats of the NFkB binding site driving the expression of the apoptotic suicide gene BAX, resulted in significant cell kill following a therapeutically relevant dose of 2-Gy X-rays [226].

#### **Heat-inducible promoters**

Hyperthermia is known to improve the response of tumours to radiation and chemotherapeutic treatment [267]. The cellular response to hyperthermia is associated with the synthesis of heat shock proteins (HSP). Strate-

gies are now being developed to combine hyperthermia with gene therapy by using the promoter of the HSP70B gene to selectively activate therapeutic transgenes following hyperthermia treatment, thereby controlling the location, duration, and level of expression of the therapeutic transgene. MRI-guided ultrasound is also being used to accurately focus gene activation [227]. The HSP70B promoter has been used to drive expression of TGF- $\beta$  in an adenoviral construct [268]; HSV-tk, where sensitivity to GCV in a mouse mammary cancer cell line was increased more than 50,000 times [228]; HSV-tk/cytosine deaminase fusion gene and a replication-competent E1B-attenuated adenoviral vector [229]; and IL-12 in combination with radiation [230, 231]. The HSP70B promoter appears to be highly active and a 200–950-fold increase in reporter gene expression was observed when additional heat shock elements (HSE) were introduced to the HSP70 promoter with a 1–2°C decrease in the activation temperature [232]. Two very elegant constructs have now been developed which incorporate elements from the HSP70 promoter. Emiliusen et al and Lipinski et al have developed constructs which incorporate effective feedback loops which can enhance transgene expression and immune response [14] or inhibit transgene expression in normal cells expressing wild-type p53 [173]. These will be discussed in more detail later. Interestingly, electromagnetic fields have been reported to activate the HSP70 promoter and could possibly be used as switches to regulate transgenes for cancer gene therapy purposes [269]. This has already been achieved with another stress-inducible promoter, Gadd153. The promoter from this gene was placed upstream of the TNF- $\alpha$  gene and delivered using magnetite cationic liposomes (MCLs). In nude mice, these constructs induced cell death throughout much of the tumour area on heating under an alternating magnetic field as a result of a 3-fold increase in TNF- $\alpha$  production compared with that of a nonheated tumour [235].

#### **Drug-inducible promoters**

As already mentioned many tissue/tumour-specific promoters are often weak and are therefore less effective than constitutive promoters. Much work has therefore focused on developing promoters which are both strong and regulatable exogenously. The use of tetracycline (tc)-responsive promoters is probably best researched. Two systems exist; the allosteric-off system or the allosteric-on system which have been described in detail by Harrington et al [78]. A tc inducible promoter has been used to drive expression of HSV-tk in breast carcinoma cells and increased the IC50 for GCV by 50 fold, although bystander effects were not enhanced [239]. Both IL-2 and IL-1 $\beta$  converting enzyme (ICE) have been shown to be tightly regulated, respectively, in human melanoma cells [240] and in a rat brain tumour model [241] in vivo using a similar approach. However, the real beauty of this system has been to selectively regulate transgene expression in

certain tissues by combining with tissue/tumour-specific promoters. A replication-incompetent adenovirus, which has the glial fibrillary acidic protein coupled to a minimal CMV promoter, has been constructed to drive expression of a tc-controlled transactivator. High levels of expression were reported in cells of glial origin and expression was tightly controlled by the addition of tc. This system is both glial-specific and allows for repression of ectopic gene expression [49]. Both a parvovirus and an adenovirus have been constructed to combine cell-type specificity with high-level regulated expression for prostate tissues using the ARR2BP promoter [57] or for hepatoma cells using a liver-specific promoter [146]. In a similar study, Rubinchik et al [242] have placed the tc transactivator gene under the control of the ARR2PB promoter and an FASL fusion gene under the control of a tc responsive promoter, within a single construct. Not only is the expression of this apoptotic related gene FASL restricted to prostate cancer cells and is regulatable by the tc analogue, doxycycline, but also, in vivo studies confirmed that systemic delivery of this vector was well tolerated at doses that were lethal for the FASL controlled by a CMV promoter. Overall, tc promoters show high-level inducibility compared to other inducible promoters and when combined with other tissue- and tumour-specific elements, they can be highly inducible and specific.

Dimerisation switch-on systems are also being developed, which make use of the immunosuppressive agent, rapamycin, and again this area has been reviewed by Harrington et al [78]. Rapamycin has been used to control regulation of adenoviral vectors where inhibition of tumour growth was seen following subcutaneous injection into the human fibrosarcoma, HT1080 [248].

As well as tc, there is now much interest in the use of estrogen response elements (EREs) which are responsive to estrogens and can be controlled by antiestrogens, such as tamoxifen. EREs have been used to drive expression of the E1a and E4 units in response to estrogens-generating conditionally replicative adenoviruses, selective for ER-positive breast cancer cells, which can also be modulated by the addition of tamoxifen [249]. When these EREs were combined with hypoxia response elements, these viruses can kill ER-positive breast cancer cells or cells growing under hypoxic conditions [150].

Lastly, a series of papers have described the use of the multidrug resistance (*mdr1*) gene promoter which harbours drug-inducible response elements to drive expression of a reporter gene [270] and TNF- $\alpha$  within a retroviral construct both in vitro in mammary and colon carcinoma cell lines [271] and in vivo in MCF-7 mammary xenografts [236]. Chemotherapy regulation by doxorubicin or vincristine showed a 25-fold increase in TNF- $\alpha$  secretion within the xenografts and was more effective in inhibiting tumour growth than constitutively TNF- $\alpha$ -expressing vectors. More recently, Walther et al [237] have shown that the *mdr1* promoter also harbours heat-inducible elements.

### STRATEGIES FOR ENHANCING THE EFFICIENCY OF WEAK TISSUE/TUMOUR-INDUCIBLE PROMOTERS

The transcriptional activity of the tissue-specific elements used as promoters for gene therapy is often too weak to generate adequate concentrations of transgene product in target cells. The dilemma is how to achieve high levels of transgene expression without compromising specificity. As mentioned previously, several elegant strategies have been developed which combine both inducible and tissue/tumour-specific elements, thereby increasing transcriptional activation while allowing tissue specificity to be retained. Furthermore, other systems have been developed which are tissue-specific but also highly active by encouraging positive feedback loops incorporating strong transcriptional activators.

In one approach, a weak tissue-specific promoter (vWF or gastrointestinal-specific sucrose-maltase) initiates transcription of both the transgene and a gene encoding a VP16-LexA fusion protein (Figure 2). This protein, in turn, interacts with LexA binding sites inserted upstream of the tissue-specific promoter, leading to transactivation and enhanced transcription. Repeated cycles of this amplification lead to very high levels (14–100 fold) of tissue-specific expression [208]. An alternative strategy using a GAL4/VP16 fusion protein to enhance the weak tumour-specific CEA promoter achieved similar amplification of the reporter genes *LacZ* or *GV16* [100]. This concept has been further developed by using a tissue-specific promoter (PSA) to drive expression of GAL4 derivatives fused with up-to-4 VP16 activation domains. By incorporating up-to-5 GAL4 binding sites upstream of the reporter gene (firefly luciferase) 800-fold amplification was achieved [272].

The *Cre/loxP* system offers a different approach to the enhancement of tissue-specific promoter activity (Figure 3). Target cells are transfected with a plasmid incorporating the therapeutic transgene separated from a strong constitutive promoter by a "stop" cassette enclosed by two *loxP* sites. Cotransfection with a site-specific *Cre* gene driven by the tissue-specific promoter generates *Cre*, which excises the stop cassette, bringing the therapeutic transgene under the control of the constitutive promoter [113]. This system has been used to achieve therapeutic levels of transgenes in mouse tumours in vivo with a high degree of tissue specificity. CEA-driven expression of *Cre* together with expression of *lox-HSV-tk* caused significant suppression of tumours in mice treated with GCV, without significant side effects [99, 104]. CEA-driven expression of *Cre* also achieved cytotoxicity in tumours in mice transfected with cytosine deaminase and dosed with the 5-FU precursor, 5-FC, and this was reflected in enhanced overall survival. Diphtheria toxin (DT) has also been used therapeutically in growth hormone (GH)-producing pituitary tumours; the DT gene was activated via the *Cre/loxP* system driven by the GH promoter [273]. *Cre/loxP* has also been used to increase HSV-*tk* transgene

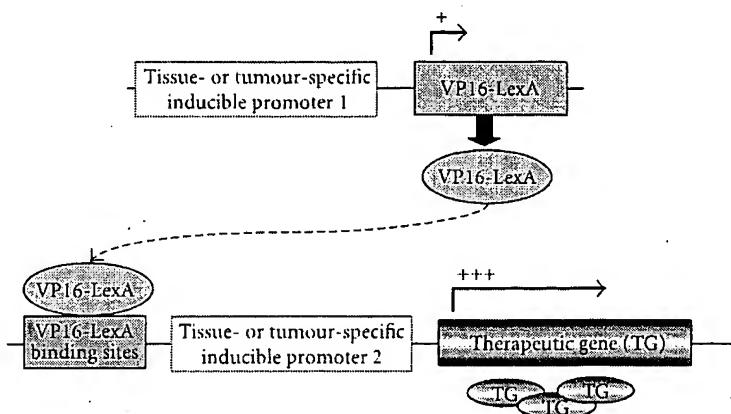


FIGURE 2. Schematic outline of a positive feedback loop utilising VP16/LexA to enhance weak promoters. Transcription is initiated by the cell-type-specific promoter in target cells which leads to expression of the VP16/LexA fusion protein. The VP16/LexA protein then interacts with the LexA binding sites upstream of a possible second cell-type-specific promoter leading to transactivation and thus enhancement of transcription. Alternatively, the VP16/LexA fusion protein could be expressed along with the transgene expressed via an IRES. Adapted from Nettelbeck et al [179].

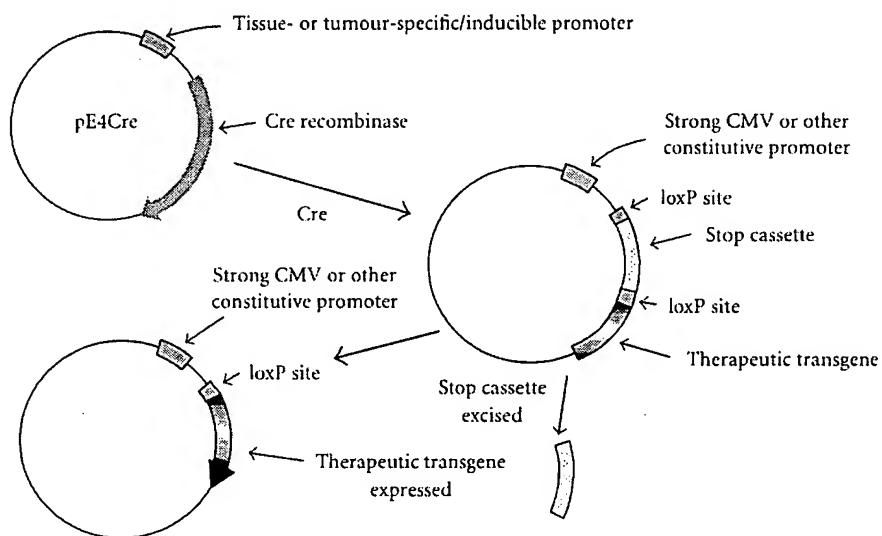


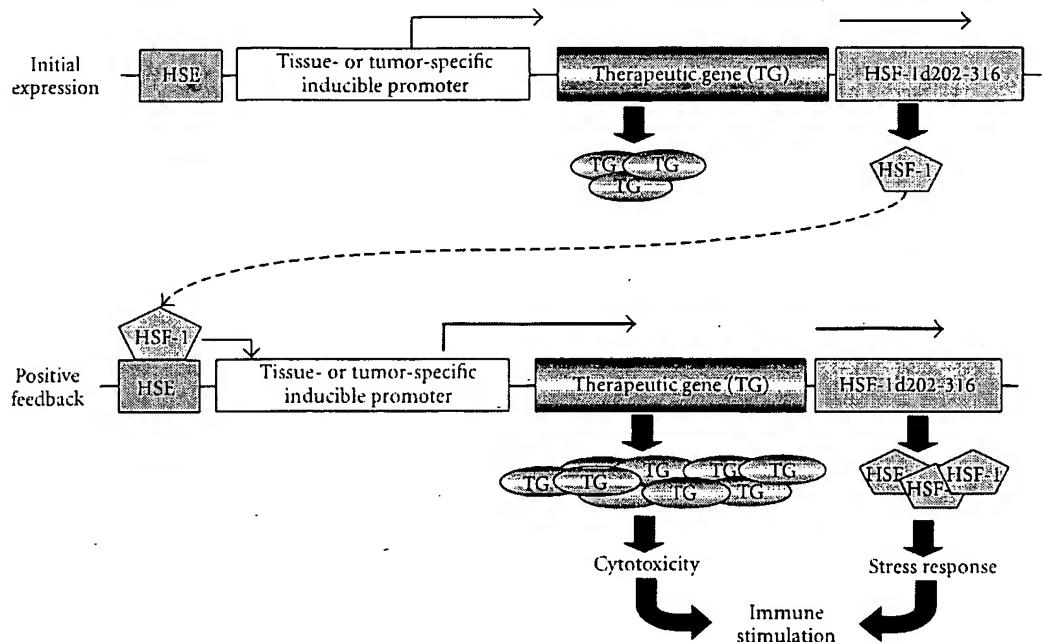
FIGURE 3. The Cre recombinase/loxP system for enhancing weak tissue or tumour-specific promoters. Cre, induced by the specific promoter excises the transcriptional stop cassette via the loxP sites. This brings the therapeutic transgene under the control of a strong constitutive promoter (eg. CMV).

expression following expression of Cre via the radiation-inducible CARG elements [209, 219].

Another approach that has a potential for tumour-specific activation incorporates specificity for mutated p53 [173]. In this study, a double recombinant adenovirus consisting of two independent expression cassettes was constructed; one controlled expression of the *E. coli* nitroreductase gene via an hsp70 promoter containing LacI binding sites, which was capable of binding a p53-inducible lac repressor, incorporated into the second expression cassette. This resulted in growth inhibition of human tumour xenografts with mutated p53 following

exposure to the prodrug, CB1954, but not in tumours with wild-type p53 status.

Lastly, high-level tissue-specific expression of a directly cytotoxic protein, gibbon ape leukemia virus hyperfusogenic envelope protein (GALV-FMG), has been achieved in melanoma cells by elegantly driving both GALV and the gene for heat shock factor 1 (HSF-1) from a human tyrosinase promoter, preferentially activated in melanoma cells [14]. Promoter activity initiates low-level expression of the toxic transgene, but also HSF-1, which binds to a heat shock binding element upstream of the tyrosinase promoter. This enhances promoter



**FIGURE 4.** A transcriptional feedback loop for tissue-specific expression of highly cytotoxic genes which incorporate an immunostimulatory component. A weak tissue- or tumour-specific promoter will initially express the therapeutic transgene (TG) at low levels, however coexpression of the heat shock transcription factor (HSF-1) will activate the heat shock element (HSE) causing progressively increased TG and HSF-1 expression via a positive feedback loop. In addition, the expression of HSF-1 causes an immune response. Adapted from Emiliusen et al [14].

activation and completes the transcriptional feedback loop (Figure 4). This strategy has achieved long-term eradication of melanomas in mice [14].

#### CONCLUSIONS AND FUTURE PERSPECTIVES

The ultimate goal of all currently used cancer therapy is to target damaging events to tumour cells while sparing normal tissues sufficiently that they can recover to an adequate functional level; this applies equally to gene therapy. However, while conventional therapies rely on one, or at the most two, targeting characteristics such as spatial deposition, biochemical pathway specificity, or cellular proliferation, there is no theoretical limit to the number of elements that can be incorporated into a gene therapy strategy to confer tumour specificity. There is also the potential for controlling temporal expression of transgene products in tumours much more precisely than the concentration of conventional anticancer agents.

The first opportunity for targeting specificity is at the level of vector delivery. There is currently an immense amount of research activity in this area (beyond the scope of this review) reflecting the recognition that advances in this process are critical to unleashing the potential of all the recent developments in transcriptional control. Direct injection systems have been important in providing the proof of principle for several gene therapy strategies, but however sophisticated these may become, the number of accessible sites will always be limited and perhaps more

importantly the crucial problem of disseminated disease cannot be addressed.

As detailed in this review, the possibilities for targeting via transcriptional control are endless. It is unlikely for most cancers that any one promoter will offer the ideal combination of interrelated characteristics: high level specificity to endogenous or exogenous factors, silence in the absence of these factors and sufficient strength to induce the therapeutic transgene many fold. In any event, the use of a single promoter/transgene combination fails to exploit one of the main advantages of gene therapy: the ability to custom design, even within a single plasmid, a combination of diverse elements that confer exquisite spatial and temporal specificity for an individual tumour type. It may even be possible using gene/protein array technology to tailor this specificity to an individual patient's tumour. It could be envisaged that promoters specific to the tumour tissue type could be combined with factors inducible by the tumour microenvironment and/or exogenous control elements (eg, radiation). This has already been successfully achieved *in vivo* by combining cell cycle and tyrosinase promoters [179], HREs and KDR/E-selectin promoters [186], and HREs and the AFP promoter [145]. Furthermore, these could be combined with elements incorporating strong promoters/enhancers in positive feedback loops giving progressively stronger and more specific transgene expression [14, 179] or by using a *Cre/loxP* system [274]. The packaging of such complex constructs, ideally within a single construct, will

require a more thorough understanding of the control domains from individual promoters such that multiple copies of the elements that confer tumour specificity can be incorporated while eliminating those sequences that permit expression in nontarget tissues.

A vital issue when considering the clinical application of any gene therapy protocol is the basal expression of the transgene. Many of the papers discussed in this review have failed to adequately assess basal-level expression in other tissues following systemic delivery in an appropriate *in vivo* model. Highly sensitive systems now exist for assessing promoter leakiness *in vitro* [42] and recent developments in imaging technology have permitted whole animal detection of fluorescent reporter gene products in small rodents [272, 275]. Although this technology is theoretically applicable in man, considerable caution must be exercised in extrapolating from animal data. It is likely in early trials that transgene activation by a given promoter combination will need to be assessed using a nontoxic reporter gene, particularly where tissue-specific promoters are to be used. This use of a surrogate marker is an additional and important advantage of gene therapy over conventional chemotherapy that can be applied even at the level of the individual patient.

Finally, the therapeutic transgene product will usually be an enzyme, so the opportunity arises to achieve additional specificity at the level of enzyme biochemistry or enzyme product characteristics. For example, reductive enzymes can metabolise bioreductive prodrugs, such as AQ4N [264, 265] or tirapazamine [276], specifically in a hypoxic environment thereby adding an additional layer of tumour specificity even after transductional and transcriptional targeting. A similarly enhanced specificity can be achieved, where the final product of the enzyme specifically sensitises hypoxic tumour cells [222, 223]. Alternatively, a powerful approach for tight regulation has been to make the activity of the apoptotic target gene, E2F1, tamoxifen-dependent by fusing it to the ligand-binding domain of the estrogen receptor; massive apoptosis was seen upon activation by tamoxifen [277].

The rate of development of gene therapy over the past decade has been dramatic and if this continues, it is reasonable to speculate that the next decade will see widespread introduction of this new technology to clinical practice. We can anticipate a level of anticancer efficacy combined with normal tissue sparing that few small molecules have yet achieved and with the current advances in systemic gene delivery this is a very real possibility. By combining the very best of cancer gene therapy strategies with current chemotherapy and radiotherapy treatment regimens, real patient benefit with reduced toxicities should be achievable.

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